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(54) **USE OF NERVE GROWTH FACTOR FOR THE MANUFACTURE OF A MEDICAMENT FOR THERAPY OF INTRAOCULAR TISSUE PATHOLOGIES**

VERWENDUNG DES NERVENWACHSTUMFAKTORS ZUR HERSTELLUNG EINES ARZNEIMITTELS ZUR BEHANDLUNG VON ERKRANKUNGEN DER INNENAUGEGEWEB

UTILISATION DU FACTEUR DE CROISSANCE DES NERFS POUR LA PREPARATION D'UN MEDICAMENT POUR LE TRAITEMENT DE PATHOLOGIES DU TISSU INTRAOCULAIRE

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- **PATENT ABSTRACTS OF JAPAN vol. 1998, no. 13, 30 November 1998 (1998-11-30) & JP 10 218787 A (OKAMOTO AKIO), 18 August 1998 (1998-08-18)**
- **LAMBIASE ET AL: "Nerve growth factor delays retinal degeneration in C3H mice" CHEMICAL ABSTRACTS + INDEXES,US,AMERICAN CHEMICAL SOCIETY. COLUMBUS, vol. 125, no. 19, 4 November 1996 (1996-11-04), XP002058562 ISSN: 0009-2258 cited in the application**

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Description

[0001] The present invention relates to the use of nerve growth factor for the therapy of intraocular tissue pathologies. More particularly, the invention relates to the use of the neurotrophin, named nerve growth factor (NGF), for the therapeutic treatment of the eye internal structures, as sclera, choroidea, ciliary bodies, crystalline lens, vitreous body, retina and optic nerve, by a topical administration over the ocular surface, i.e. as collyrium or ophthalmic ointment.

[0002] The nerve growth factor (NGF) is the chief molecule of a complex neurotrophin family, and is well known for its trophic, tropic and differentiating activity on cholinergic neurons of the central nervous system and on the sympathetic peripheral system. NGF is produced by various mammalian tissues, included humans, and is released in the blood stream in greater amounts during the growth and differentiation of the nervous system. Biological, biochemical and molecular studies carried out on *in vitro* cellular systems have pointed out high sequence homology between murine and human NGF. Furthermore, in humans and other mammalians NGF is normally contained both in the cerebrospinalis liquor and blood stream at concentrations of about 10-15 pg/ml. The value increases during some inflammatory pathologies (autoimmune and allergic diseases, etc.), whereas it decreases in others (diabetes).

[0003] NGF has been discovered by Prof. Rita Levi-Montalcini, at the Zoology Institute of the Washington University of St. Louis (Levi-Montalcini R., Harvey Lect., 60:217, 1966), and its discovery represented a remarkable advance in the study of the growth and differentiation mechanisms of the nerve cell, as NGF is able to affect the development and preservation of the biological functions of the neurons and their regeneration. In 1986 the Nobel Prize for Medicine and Physiology was awarded to Prof. R. Levi-Montalcini for the discovery of this molecule and the characterization of its biological function both in peripheral and in central nervous system

[0004] Various experimental studies both *in vitro* and *in vivo* have demonstrated the physiopathological importance of NGF in preventing neuronal injury of surgical, chemical, mechanical and ischemic origin, thereby making it the ideal candidate for use in the therapy of various pathologies affecting both the peripheral and central nervous systems (Hefti F., J. Neurobiol., 25:1418, 1994; J. Fricker, Lancet, 349:480, 1997). In fact since some years ago clinical tests are being carried out on subjects affected by Parkinson's Disease and Alzheimer's Disease by intracerebral administration of murine NGF (see, for example, Olson L. et al., J. Neural Trans.: Parkinson's Disease and Dementia Section, 4:79, 1992). Results of these experiments confirmed the observations obtained from animal models and pointed out the absence of possible side effects following the administration of murine NGF. This behaviour has been confirmed more recently for recombinant human NGF (Petty B.G. et al., Annals of Neurobiolgy, 36:244-246, 1994).

[0005] Studies on the characterization of biological, biochemical, molecular, pre-clinical and clinical effects of NGF almost exclusively have been carried out using NGF isolated from submandibular glands of adult rodents; therefore available data concern mostly murine NGF. The biochemical properties of the latter, particularly, have been described in a study published in 1968 (Levi-Montalcini R. and Angeletti P.U., Physiological Reviews, 48:534, 1968).

[0006] The NGF contained in murine salivary glands is a 140 kdalton molecular complex, the sedimentation coefficient thereof being 7S, and it is constituted by three sub-units, α, β and γ, the second one of which represents the actual active form. The latter, called βNGF, whose sedimentation coefficient is 2.5S, is usually extracted and purified according to three not very different techniques (Bocchini V., Angeletti P.U., Biochemistry, 64:787-793, 1969; Varon S. et al., Methods in Neurochemistry, 203-229, 1972; Mobley W.C. et al., Molecular Brain Research, 387:53-62, 1986).

[0007] The so obtained βNGF is a dimer of ~ 13.000 dalton, consisting of two identical chains of 118 amino acids. Each chain is stabilised by three disulphide bridges, while non-covalent bonds assure the stabilisation of the dimeric structure. The molecule is very stable and is soluble in almost all solvents, both aqueous and oily, maintaining unchanged its biochemical characteristics and biological activity. Further details about the structure, physical and biochemical properties of the molecule are reported in Green, L.A. and Shooter, E.M., Ann. Rev. Neurosci., 3:353, 1980.

[0008] Recently the structure of βNGF has been further disclosed by means of crystallographic analysis. The analysis pointed out the presence of three anti-parallel filament pairs, having a β-type secondary structure, forming a flat surface along which the two chains join together resulting in the active dimer. On these βNGF chains the presence of four "loop" regions has been showed, wherein many variable amino acids are included. These variable amino acids are probably responsible for receptor recognition specificity.

[0009] The biological effect of NGF is mediated by two receptors present on the surface of the corresponding target cells. The existence of various antibodies that selectively inhibit the NGF biological effect has allowed an accurate characterization and modulation of the activity thereof, both in cellular systems and *in vivo*.

[0010] More recently human NGF has been synthesized using genetic engineering techniques (Iwane et al., Biochem. Biophys. Res. Commun., 171:116, 1990) and small amounts of human NGF are commercially available too. However, direct experimentation has shown that the biological activity of human NGF is very low when compared to murine NGF. Furthermore it is to be pointed out that almost all of data available concerning human NGF, both *in vivo* and *in vitro*, have been obtained using murine NGF and no undesirable side-effects resulting from the murine origin of the molecule have ever been detected.

[0011] Studies carried out on animal models starting in the 90's suggested a possible involvement of NGF in ocular

pathologies. As far as the scientific works are concerned, the reports published in the ophthalmic field exclusively refer to the use of NGF in retinal affections and in affections of the optic nerve, i.e. on the nervous tissue.

[0012] Particularly, it has been reported that the intraocular administration of NGF to animal models is effective in enhancing the survival of retinal ganglion cells following acute retinal ischemia (Siliprandi R. et al., Inv. Ophthalmol. Vis. Sci., 34:3232, 1993) and following optic nerve transection (Carmignoto G. et al., J. Neurosci., 9:1263, 1989). More recently the NGF administration by intravitreous or also retrobulbar injection proved to be effective in a mouse retinal degeneration model, which is similar to human retinitis pigmentosa (Lambiase A. and Aloe L., Graefe's Arch. Clin. Exp. Ophthalmol., 234:596-S100, 1996), and in a rabbit retinal damage model resulting from ocular hypertension (Lambiase A. et al., Graefe's Arch. Clin. Exp. Ophthalmol., 235:780-785, 1997).

[0013] Such experimental studies showed that the local administration of NGF is effective for preventing or at least delaying the death of retinal ganglion cells and photoreceptors resulting from the above pathologies. In addition no side effects during the animal treatments have been reported. However, it is to be pointed out that in all the scientific publications referred to above, NGF is administered to the internal ocular tissue by intravitreous injection or by retrobulbar injection.

[0014] In agreement with the foregoing, the PCT patent application WO 90/12590 discloses a method of treating open-angle glaucoma and a method of treating retinal disease, both consisting of providing to the trabecular meshwork of the eye or to the optic cup, respectively, a substance chosen from a wide range of biologically active proteins, among which also NGF is cited. In both cases, an effective amount of the substance must be provided to the affected ocular tissue by site specific delivery means, the preferred method of delivery being by microinjection.

[0015] With specific reference to the disorders affecting the exposed ocular surface, i.e. corneal and conjunctival diseases, EP-A-0312208 discloses gel formulations for use in the treatment of epithelial lesions and epithelial pathologies in general, including lesions and pathologies of the ocular surface. The said formulations contain an active ingredient which may be indiscriminately chosen among the various molecules whose name contains the expression "growth factor". Although the description is exclusively concerned with the epidermal growth factor (EGF) as the preferred active ingredient, and although activity data (*in vitro*) and formulation examples are given only for EGF, other growth factors are mentioned as well, such as FGF (fibroblast growth factor), PDGF (platelet-derived growth factor), TGF-α (transforming growth factor) or the NGF itself. The said growth factors are apparently presented as a family of molecules having equivalent characteristics and biological activity as EGF. As a matter of fact, at the current state of the knowledge, it is undisputed that the said growth factors have different specific targets and that they often have conflicting effects, so that they are not considered as biologically equivalent to each other.

[0016] The use of NGF for external ophthalmic administration, for example in the form of collyrium or ointment, is actually described in the PCT patent application WO 98/48002, having the same inventor as the instant application. The experimental work therein reported proves that topically administered NGF is suitable for a successful treatment of ocular surface pathologies (i.e., affecting cornea and conjunctiva) both of acquired and congenital type and, particularly, of various dystrophic or neurodystrophic pathologies for which therapeutic treatments did not exist previously. The discovery of the presence of NGF and of its high affinity receptor (TrkA, tyrosine kinase A) in corneal tissue, made by immuno-hystochemical techniques, was the preliminary step for such innovative result. Evidently the expression of the NGF high affinity receptor is an essential prerequisite for NGF to exert its therapeutic activity.

[0017] In the frame of the studies which led to the present invention it has been found, by both immuno-hystochemical and immunofluorescence techniques (Lambiase et al., J. Allergy Clin. Immunol., 100:408-414, 1997) and, in addition, by biomolecular techniques for the *in situ* identification of the NGF mRNA (Micera A. et al., Archives Italiennes de Biologie, 133:131-142, 1995), that all cells of the sclera, crystalline anterior capsule, ciliary body epithelium, the optic nerve fibers, the retinal ganglion cells, the retinal pigmented epithelium cells and some choroidea cells not only express the high affinity receptor for NGF but are also able to produce this neurotrophin (not yet published data). These experimental data result in various implications. On the one hand NGF, released from cells of various ocular tissues, should exhibit a trophic and physiopathological activity in all the ocular regenerative mechanisms; on the other hand, various pathologies of trophic, degenerative or immune type should recognise the failed release of NGF as a fundamental etiologic step.

[0018] Furthermore, as the effects observed after the administration of exogenous NGF are present at almost physiological concentrations (in the order of about a few micrograms), it is conceivable that in some ocular affections the reduction of local NGF levels under the threshold value suitable to assure the tissue integrity can be a possible physiopathogenetic mechanism. Such a pathogenetic hypothesis is confirmed by some published data concerning the effects of NGF deprivation. The latter induces, both *in vitro* and *in vivo*, the death of various cell populations and the exacerbation of tissue damages of chemical, physical, infective or degenerative type (Aloe L., Int. J. Devl. Neuroscience, volume 5 (4), 1987; Lambiase A. and Aloe L., cited above; Lambiase et al., Graefe's Arch. Clin. Exp. Ophthalmol., 1997, cited above).

[0019] Although the above results allow to hypothesise a therapeutic activity of NGF also on ocular structures and tissues different from those already reported in the literature, and specifically on sclera, ciliary bodies, crystalline, vitreous body and choroidea, there is the problem of an easy administration of the active principle to the involved tissues. Contrary

to the case considered in the PCT patent application WO 98/48002, referring to corneal and conjunctival pathologies, in this case tissues within the eyeball are involved.

[0020] The possibility of administering an ophthalmic therapeutic agent by the external ophthalmic route, i.e. in the form of collyrium or ointment, represents a remarkable benefit in comparison with the administration through the topical parenteral route, e.g. by retrobulbar or intravitreous injection. In fact the use of these latter techniques involves the risk for various complications, reported in literature, such as the ocular bulb perforation, infections, haemorrhages and lesions of anatomical structures during injection. Such complications can occur even more frequently during the treatment of chronic pathologies, and can lead to the unfeasibility of the therapy due to the inversion of the risk/benefit ratio.

[0021] It has now surprisingly been found that by administering NGF in the form of collyrium, an increase of the neurotrophin levels in all ocular tissues, including those internal to the ocular bulb, is obtained. As it will be illustrated in detail in the following experimental report, the passage of NGF from the ocular surface, where it is administered, to internal ocular tissues, has been shown using both an autoradiographic method (Levi-Montalcini, R. and Aloe L., Proc. Natl. Sci. USA 82:7111-7115, 1985), and an immunoenzymatic assay (Bracci-Laudiero, L. et al., Neurosci. Lett., 147: 9-12, 1992). The application of the latter method on rabbits treated by conjunctival instillation of a NGF-containing saline solution has caused, one hour after the administration, an increase of NGF concentration in all the examined ocular tissues. The NGF level is reduced to initial levels after 6-8 hours. This effect allows NGF to perform its therapeutic activity also in tissues not directly involved by a superficial administration. This aspect is innovative not only with reference to the ophthalmic pathologies for which till now the NGF therapeutic activity had not even been hypothesized, but also for the retinal and optic nerve pathologies, wherein the potential activity of NGF has been already reported, but it was not possible to administer the drug in a ready and safe way without risks and drawbacks for the patient.

[0022] The only known publications wherein a topical ophthalmic administration of NGF is disclosed, for the therapy of glaucoma and optic nerve affections, are the Japanese patent application JP 10 218787 and the EP-A-0958831, having closely related subject-matter, both designing Okamoto as the inventor. Apparently, NGF or a derivative thereof, in admixture with, or as an alternative to another neurotrophin, brain-derived neurotrophic factor (BDNF) or a derivative thereof, are proposed for the above therapeutic purposes for administration to the eye by any route, including the external application as eye drops or by means of a medicated contact lens. In spite of the fact that the description of EP-A-0958831 recites extremely wide ranges of concentrations for the active neurotrophin in the medicinal preparation by which it is to be administered, the only actual data available, given in the examples, recite NGF concentrations, in an ophthalmic solution, of 0.04 and 0.02 μg/ml. Any person skilled in the art who tried to carry out the above teachings in an *in vivo* test, thereby administering such concentrations of NGF by the external ophthalmic route, would not be able to detect any effect on the clinical conditions concerned. Also any experimental test on animals would confirm that NGF, administered on the eye surface as eye drops at the concentrations specified above, would not pass in a detectable amount from the ocular surface to the internal tissues.

[0023] Accordingly, the present invention specifically provides the use of nerve growth factor (NGF) for the production of an ophthalmic preparation for administration over the intact ocular surface for the therapy and/or the prophylaxis of pathologies affecting the sclera, ciliary bodies, crystalline lens, retina, optic nerve, vitreous body and/or the choroidea, wherein said ophthalmic preparation contains from 200 to 500 μg/ml of NGF. Specifically said NGF-containing ophthalmic preparation is in the form of a solution or a suspension, an ointment, a gel or a cream in a pharmaceutically acceptable carrier, which is tolerated by the eye and compatible with the active principle. It is also possible to conceive particular routes of ophthalmic administration for delayed release, as ocular erodible inserts, or polymeric membrane "reservoir" systems to be located in the conjunctival sac. Alternatively NGF, or a preparation containing it, can be added to a local bandage together with a therapeutic contact lens.

[0024] As already pointed out said ophthalmic preparation is suitable for the therapy and/or the prophylaxis of pathologies affecting the sclera, ciliary bodies, crystalline lens, retina, optic nerve, vitreous body and choroidea, said affections having trophic, post-traumatic, infective, post-surgical, autoimmune, dystrophic, degenerative or post-inflammatory origin, or being originated by laser treatment. As it will be demonstrated by the experimental data reported below, the external topical administration of NGF proved, among other things, to be able to repair scleral lesions of traumatic or immune origin, to cause an increase of aqueous humour production, restoring the intraocular pressure in pathologies characterised by hypotonia and resulting in bulbar phthisis, and to prevent and delay the formation and progression of crystalline lens opacity (cataract). As to the retinal pathologies, the NGF administration by application over the ocular surface induces an increase of nervous fiber thickness, a survival of retinal ganglion cells, photoreceptors, and pigmented epithelium during degenerative, ischemic, traumatic pathologies and when damages from ocular hypertonia are present. As to the optic nerve, the effects obtained are an improvement of visual evoked potentials (VEP), of visual field and of the survival of nervous fibers when traumatic, ischemic, pressor and degenerative pathologies occur. Finally, as to choroidea the NGF administration by external ophthalmic application causes a reduction of choroidea inflammatory processes and reduces the number of mobile vitreous bodies. It is to be pointed out that many of these disorders are hardly therapeutically treated, or they lack any effective treatment.

[0025] The possibility that nerve growth factor could exhibit a biological activity on internal tissues of the ocular bulb

following an external local administration was hardly predictable mainly considering that, as pointed out before, NGF is a quite big molecule (26,800 dalton) with a complex structure. In order that an exogenous molecule can exert its activity on deep ocular tissues, it is necessary that, once it has been instilled over the eye surface, the molecule pass through the lacrimal layer, the cornea, the aqueous humour and the vitreous body so to be distributed within all the tissues. According to the current practice no molecules (particularly antibiotics or cortisone molecules) which are able to reach the crystalline lens, vitreous body and retina at therapeutically effective concentrations are presently available. For the above reasons in all the known studies on the utilisation of NGF for ocular pathologies, only the intraocular administration route was used.

[0026] In effect NGF, although having a complex structure and high molecular weight, includes both hydrophilic and hydrophobic groups which allow it to pass through the homologous (lipid and hydrophilic) anatomical barriers. Furthermore it is a basic characteristic of NGF that once it has reached the target organs, also at very low but yet biologically active concentrations, it is able to stimulate tissue to produce endogenously the NGF. The presence of an endogenous fraction of NGF is clearly suggested by experimental results on the passage of NGF through tissues. These results furthermore show that a concentration gradient is not maintained from the external surface to deeper eye tissues, as it would be conceivable in the presence of a simple diffusion mechanism through the tissues.

[0027] In order to produce the preparation according to the present invention, suitable procedures for the NGF extraction and purification are reported in the previously cited references. The technique according to Bocchini and Angeletti, herein briefly reported, has been used for the experiments of the present invention. Submandibular glands of adult male mice are collected in a sterile way and tissues thereof are homogenised, centrifuged and dialysed; then the obtained suspension is passed through subsequent cellulose columns, whereon NGF is adsorbed. Then NGF is eluted with a buffer containing 0.4 M sodium chloride. The obtained samples are analysed spectrophotometrically at a 289nm wavelength to identify the NGF containing fractions. These fractions are dialysed and the NGF is lyophilised in a sterile way and stored at -20°C in freezer.

[0028] A medicament according to the invention suitable for administration over the intact ocular surface contains, alone or optionally in association with one or more other active principles, from 200 to 500 μg/ml of NGF. In the case the product is in the form of an aqueous solution (collyrium), the concentration of NGF is preferably between 200 and 250 μg/ml. A specific formulation suitable in the form of collyrium contains, for example, 200 μg/ml of NGF in physiological solution containing 0.9% of sodium chloride, or in balanced saline solution (BSS^{R}); in both circumstances the solution is isotonic with the tear fluid and therefore well tolerable by the eye. However it is also possible the use of hypotonic solutions.

[0029] The NGF contained in the saline solution can be present alone or in association with other biologically active molecules, and/or conjugated with carrier molecules (as, for example, transferrin). In order to further enhance its passage through ocular surface, other excipients selected from those conventionally used according to pharmaceutical techniques, for example to buffer the solutions or suspensions, to stabilise the active principle and make the preparation well tolerable can be added. Specifically buffers should keep pH between 4 and 8. For example the above reported sodium chloride solution can be buffered using any of the buffers well known in the pharmaceutically field as suitable for ophthalmic use, among which phosphate or trizma (tri-hydroxymethyl-aminomethane) buffers, so as to have a physiological pH, i.e. 7.0-7.4, maintaining simultaneously a physiological osmolarity (295-305 mOsm/l).

[0030] The tolerability can be further enhanced using excipients like polysorbate 80 (or Tween 80), dextran, polyethylene glycol (for example PEG 400) and like. The formulation can contain also viscosity-enhancing agents like hyaluronic acid, methylcellulose, polyvinylalcohol, polyvinylpyrrolidone and others, in order to enhance the ocular bioavailability, stability and tolerability of the active principle. The ocular bioavailability of NGF can be further enhanced by using compounds that ameliorate the corneal permeation of the drug as, for example, dimethylsulfoxide, taurocholates, membrane phospholipids and various surfactant agents suitable for ophthalmic use. In addition, to prevent contamination, a preservative agent having antimicrobial activity can be added to the formulation.

[0031] Agents like carboxymethylcellulose or the like can be added to products to be administered in the form of suspension. If it is desired to use the formulation in the form of ointment, gel or ophthalmic cream, the NGF carrier could be polyethyleneglycol, polyacrylate, polyethyleneoxide, fatty acid and alcohol or lanolin, paraffin and similar products.

[0032] As already pointed out the therapeutic activity of nerve growth factor on ocular tissues other than the superficial ones (cornea and conjunctiva), as well as on the retina, and on the optic nerve, has not been previously disclosed, neither when it is administered by intraocular injection or when it is administered in formulations in the form of collyrium or ointment.

[0033] Some experimental results, obtained within the frame of the present invention, including clinical data concerning therapeutic applications on humans, are reported below merely for exemplary purposes.

Studies on the passage of NGF through the ocular tissues

[0034] In a first set of tests to study the passage of NGF intraocularly from the external surface over which it was

administered, the above mentioned autoradiographic method has been used for a group of six rabbits. Each of the animals was administered with one collyrium drop (50 μl) containing 10 μg of I^{125} labeled NGF (concentration: 200 μg/ml) by instillation in the conjunctival fornix.

[0035] Murine NGF purified according to the previously described method and subsequently conjugated to Na-I^{125} (Amersham Italia, IMS30, 1mCi) according to the chloramine T method (Lapack PA. Exp. Neurol. 124:1620, 1993) has been used. The amount of labeled NGF has been determined by chromatography using a Sephadex G-25 column. The amount of the I^{125} labeled product collectible by precipitation was between 90% and 95%, showing that most of the radioactive product was bonded to NGF. The specific activity of NGF-I^{125} was between 1 and 1.5 Ci/μmol.

[0036] Two hours following the administration of the labeled NGF the animals were sacrificed and eyes enucleated and fixed in 4% paraformaldehyde over 48 hours. Then samples, after incubation in 30% sucrose over 24 hours, were cut with a cryostat to 15μm thick sections. Sections were mounted on histology gelatinous slides, immersed in photographic emulsion (Ilford K2) and incubated over 4 weeks at 4°C. The sections were successively dehydrated using ethanol, mounted on DPX after treatment with xylene and examined with Zeiss optical microscope.

[0037] This experiment showed that labeled NGF, after its administration over the ocular surface, was able to penetrate into the eye and bond with cells of various tissues contained in the posterior segment and crystalline lens inducing the expression of the specific receptor.

[0038] In a second set of tests, using the above described immunoenzymatic method, the quantitative levels of NGF in various ocular tissues after the administration by instillation of a drop of murine NGF in the conjunctiva fornix were determined. In all 24 rabbits were used, six thereof were sacrificed immediately to determine the baseline values of NGF concentration in various ocular tissues. Remaining animals were sacrificed after 1 (6 rabbits), 2 (6 rabbits) and 8 hours (6 rabbits) following the administration of the collyrium.

[0039] In all the cases the eyes were enucleated and the different tissues (cornea, sclera, aqueous, iris, crystalline lens, retina, choroidea, optic nerve) were sectioned. The tissues were weighed, sonicated (using Braun B Sonicator) in a buffered protein matrix containing protease inhibitors (extraction buffer). The thus obtained homogenate was centrifuged (x 10000 rpm for 20 minutes) and the surpernatant was used to determine the levels on NGF by immunoenzymatic method (ELISA). This technique is extremely sensitive and NGF specific, and it is able to detect concentrations up to 5 pg/ml. Goat anti-NGF polyclonal antibody, diluted in 0.05 M carbonate buffer, pH 9.6, was used as first antibody. As control, for the determination of unspecific signal, purified goat immunoglobulins were used.

[0040] Solutions containing primary antibody and control immunoglobulins were placed in parallel on polystyrene 96-well plates. Then the plates were incubated for 12 hours at room temperature and then the unspecific sites were blocked using a solution containing carbonate buffer plus 1% BSA. Further to plate washings with 50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 0.5% gelatine, and 0.1% Triton X-100, NGF samples and standard solutions were suitably diluted with 50 mM Tris-HCl, pH 7.2, 400 mM NaCl, 4 mM EDTA, 0.2 mM PMSE, 0.2 mM benzethonium chloride, 2 mM benzimidine, 40 U/ml aprotinin, 0.05% sodium azide, 2 % BSA and 0.5 % gelatine. After triplicate distributions of standard solutions and samples of NGF in an amount of 50 μm/well, the plates were incubated with the secondary antibody: 4 mU/well of anti-β-galactosidase (Boerhinger Mannheim, Germany) for 2 hours at 37°C. Then, after the washings, 100 μl/well of a solution containing 4 mg of β-galactosil-chlorophenol red (Boerhinger Mannheim Germany)/ml of 100 mM HEPES, 150 mM NaCl, mM $MgCl_2$, 0,1% sodium azide and 1% BSA solution were distributed.

[0041] After the incubation of the chromogen for a period of two hours at 37°C the optical density at wavelength of 575 nm was determined using an ELISA reader (Dynatech). The concentration values of NGF standards and samples were calculated after subtraction of background values due to unspecific bonds. Data reported as pg/ml or pg/g are referred to fresh weighed tissue. The results, summarized in the following Table 1, show that: after one hour from the collyrium administration the NGF concentration values are increased in all the intraocular tissues, these values are maintained high, although reduced, after 2 hours, and after 8 hours they are again the same as the baseline ones.

Table 1 NGF concentrations in various ocular tissues after NGF administration in the form of collyrium (NGF pg/g of tissue)

HRS	SCLERA	CHOROIDEA	RETINA	OPTIC NERVE	CRYSTALLINE LENS	VITREOUS BODY
0	100 ± 50	960 ± 400	83 ± 50	83 ± 50	100 ± 15	10 ± 4
1	1414 ± 30	2800 ± 700	484 ± 70	1195 ± 180	200 ± 30	73 ± 12
2	694 ± 150	1813 ± 900	322 ± 100	342 ± 115	150 ± 20	20 ± 5
3	200 ± 100	100 ± 500	150 ± 70	130 ± 100	110 ± 20	10 ± 5

Studies on the effect of NGF administration in the form of collyrium for scleral pathologies

[0042] Presently no therapeutic treatments effective to induce reparations for both traumatic and immune or infective scleral lesions are known. In the case of autoimmune pathologies the formation of malacic sclera zones (scleromalacia) occurs which tend progressively to enlarge and become deeper with possible bulb perforation. Surgical treatment is the unique usable therapy and it includes the coating of damaged or malacic zone with a layer of human stored sclera or other biocompatible human tissues. However, in the case of immune affections, recidivations of the scleral pathology often occur.

[0043] In the studies in connection with the present invention the effect of external administration in the form of collyrium of murine NGF (2.5S), at a concentration of 250 μg/ml in balanced saline solution, was evaluated for 4 cases of scleral lesions, 2 of which post-traumatic and 2 scleromalacic due to autoimmune diseases (reumatoid arthritis, AR and systemic lupus erythematosus, respectively). Therapeutic protocol included the daily instillation of one or two drops of preparation in the following way: during the first two days every two hours, six times a day up to the second day from the complete sclera reparation and four times a day during the following fifteen days. The therapy, once interrupted, should be immediately restarted if initial signals or symptoms of recidivations of scleral pathology are present.

[0044] All the patients within two weeks from the beginning of the treatment with NGF showed clear signals of recovery. None thereof showed occurrence of local or systemic side effects during or after the treatment. Obtained data are summarised in the following table.

Table 2 Effect of treatment with NGF in the form of collyrium for scleral pathologies

Pat. No.	Pathology	Age years sex	Occurrence	Extension	NGF Treatment	Outcome	Follow up
1	perforating trauma	35, F	4 days	4 mm	21 days	recovery	8 months
2	perforating trauma	42, M	5 days	6 mm	25 days	recovery	6 months
3	scleromacia in AR	55, F	30 days	5 mm	20 days	recovery	10 Months
4	scleromacia in LES	42, M	25 days	4 mm	17 days	recovery	8 months

Studies on the effect of NGF administration in the form of collyrium for the production of aqueous humour

[0045] The effect of topical administration of NGF on the production of aqueous humour was determined first on a set of 6 rabbits with normal intraocular pressure. Using a tomography based method including a probe in anterior chamber of eye which is able to evaluate the modifications in the production of aqueous humour, it was recognised that the administration of NGF in the form of collyrium every two hours at a concentration of 200 μg/ml, in balanced saline solution, induces a five-fold increase in the production of aqueous humour. Such an increase is maintained during all the period of treatment.

[0046] On the base of the results obtained on animal model three patients with remarkable ocular hypotonia were treated, two of which showed hypotonia following surgical treatments (2 eyes) and the other as a result of a recurrent chronic uveitis. Due to very low intraocular pressure values (< 4 mm Hg), rapidly medical conditions were degenerating to bulb phthysis. The therapeutic protocol included the instillation of one or two drops of NGF preparation (200 μg/ml) in balanced saline solution every two hours until a successful clinical outcome.

[0047] All the treated patients exhibited clear symptoms of recovery within two weeks from the beginning of NGF treatment, the intraocular pressure values being again between 8 and 12 mm Hg within 4 weeks. No patient showed the occurrence of local or systemic side effects during the treatment or the following period. Obtained data are summarised in the following table.

Table 3 Effect of the administration of NGF in the form of collyrium on production of aqueous humour

Pat. No.	Pathology	Age years Sex	Occurrence	NGF Treatment	Outcome	Follow up
1	vitrectomy	40, M	30 days	21 days	9 mm Hg	7 months
2	vitrectomy	53, F	25 days	25 days	10 mn Hg	11 months

Table continued

Pat. No.	Pathology	Age years Sex	Occurrence	NGF Treatment	Outcome	Follow up
3	chronic uveitis	45, F	40 days	20 days	12 mm Hg	10 months

Studies on the effect of NGF treatment in the form of collyrium for the cataract prevention

[0048] Because it has been recognised that cells of the crystalline lens capsule express the receptor with high affinity for NGF and simultaneously produce this neurotrophin, it was studied whether variations of local values of NGF resulted in formation of crystalline lens opacity (cataract, a process usually related to senescence phenomena, diabetes, steroid treatment, traumas or physical stresses) and whether the external administration of NGF could prevent the formation or progression thereof.

[0049] To demonstrate the activity of NGF firstly a model of *in vitro* formation of cataract was used. In the study 18 crystalline lenses from adult rats were collected and incubated in a xilose containing medium. Then 6 crystalline lenses were treated by the addition to the medium of amounts of murine NGF variable between 1 and 300 pg/ml, 6 crystalline lenses were treated by the addition of amounts of anti-NGF antibody between 500 and 1000 μg and the remaining were left untreated as control. After 48 hours from the beginning of the culture it was clear that 6 crystalline lenses treated with anti-NGF antiboby exhibited almost full cataract, whereas 6 control crystalline lens exhibited cortical cataract with poor involvement of nucleus of crystalline lens. The remaining 6, treated with NGF, exhibited only rare opacity traces, the best response being obtained with NGF concentration of about 200 pg/ml in culture medium.

[0050] To confirm the in vivo NGF activity in preventing the cataract occurrence a cataractogenesis animal model involving a diet including 30% glycerol was used. All the animals (100%) subjected to this diet exhibit a cataract within the 44th day. A group comprising ten animals was treated by three daily administrations of NGF in the form of collyrium at a concentration of 200 μg/ml in balanced saline solution, a second group again comprising ten animals was subjected to a treatment with anti-NGF antibodies injected in the anterior camera and the last group of animals was treated with saline solution in drops and was used as control.

[0051] All the rats of the group treated with anti-NGF antibody developed a cataract within the 30th day from the beginning of the experiment; all the rats treated with saline solution developed a cataract within the 45th day from the beginning of the experiment, whereas only two rats of the group treated with NGF (20%) developed a cataract within the 45th day.

Studies on the effect of NGF in the form of collyrium for retinal pathologies

[0052] To evaluate the efficacy of the NGF administration on ocular surface for retinal pathologies in a first step experiments disclosed in literature carried out on animal models were repeated using, in addition to intravitreous or retrobulbar administrations, the administration of NGF in the form of collyrium, every two hours, at a concentration of 250 μg/ml in saline balanced solution. In all the experiments both in retinal ischemic and ocular hypertonia damage NGF administered in the form of collyrium exhibited the same activity as when administered by other administration routes.

[0053] On the basis of the results obtained from animals a total of 7 patients were treated, three of which suffering from retinitis pigmentosa, one from macular foramen, two for senile atrophic maculopathy and one for myopic retinopathy. The therapeutic protocol included the instillation of one or two drops of NGF in the form of collyrium at a concentration of 250 μg/ml in balanced saline solution every two hours for 4 weeks. Treatment results were evaluated by objective examination, electroretinogram (ERG), blood flow from central retina artery (evaluated by OBF), contrast sensitivity, thickness of the layer of nervous fibers (evaluated by OCT), microperimetry and visus.

[0054] After 4 weeks of treatment all the considered parameters resulted remarkably better; particularly an improvement of ERG, blood flow, contrast sensitivity values and an increase of nervous fibers, microperimetry and visus were detected. Obtained data are summarised in the following Table 4.

Table 4 Effect of treatment with NGF in the form of collyrium on retinal pathologies

Pat. No.	Pathology	Age years Sex	Treatment form	Treatment with NGF	ERG[1]	OBF[2]	Contrast sensitivity	OCT[3]	Microperimetry	Visus
1	Retinitis pigmentosa	35, F	collyrium	4 weeks	++	+	++	+	+	++
2	Retinitis pigmentosa	40, F	collyrium	4 weeks	++	+/-	++	+	+	++
3	Retinitis pigmentosa	32, M	collyrium	4 weeks	+++	++	++	+	++	++++
4	macular foramen	55, F	collyrium	4 weeks	+	+	+	+++	+++	+++
5	senile macular degeneration	70, F	collyrium	4 weeks	+	+/-	+	++	+++	+++
6	senile macular degeneration	73, M	collyrium	4 weeks	+/-	+/-	+	++	++	+
7	miopic retinopathy	26, M	collyrium	4 weeks	+	+	+	++	+++	+++

The values are expressed as improvement with reference to the values before the treatment with NGF: "-" = constant or worsening; "+/-" = improvement < 10 %; "+" = improvement between 11 % and 25 %; "++" = improvement between 26 % and 50 %; "+++" = improvement between 51 % and 75 %; "++++" = improvement higher than 75 %;

1) ERG = electroretinogram;

2) OBF = blood flow of central retinal artery;

3) OCT = thickness of the nervous fiber layer.

Studies on the effect of NGF in the form of collyrium for optic nerve pathologies

[0055] To evaluate the efficacy of the administration of NGF on the ocular surface in retinal pathologies in a first step experiments carried out on animal models already disclosed in literature were repeated using, in addition to already disclosed intravitreous or retrobulbar administrations, also the administration of NGF in the form of collyrium, every two hours, at a concentration of 250 μg/ml in saline balanced solution. In all the experiments of crash and ischemic injury of optic nerve the NGF administered in the form of collyrium exhibited the same activity as when administered using other administration routes.

[0056] On the base of results obtained from animals a total of 7 patients were treated, three of which suffering from low pressure glaucoma, two from retrobulbar neuritis and two from ischemic optic neuritis. The therapeutic protocol included the instillation of one-two drops of NGF in the form of collyrium at a concentration of 200 μg/ml in balanced saline solution every two hours for 4 weeks. Treatment results were evaluated by objective examination, visual evoked potentials (VEP), blood flow from central retinal artery (evaluated by OBF), contrast sensitivity, thickness of the layer of nervous fibers (evaluated by OCT), microperimetry, visual field and visus.

[0057] After 4 weeks of treatment all the considered parameters resulted remarkably better; particularly an improvement of VEP, blood flow, contrast sensitivity values and an increase of nervous fibers, microperimetry, visual field and visus were detected. The obtained data are summarised in the following Table 5.

Table 5 Effect of treatment with NGF in the form of collyrium on optic nerve pathologies

Pat. No.	Pathology	Age years Sex	Treatment with NGF	VEP[1)]	OBF[2)]	Contrast sensitivity	OCT[3)]	Microperimetry	Visual field	Visus
1	normal pressure glaucoma	45, F	4 weeks	+++	++	++	++	++	++	++
2	normal pressure glaucoma	37, F	4 weeks	++	+	+	++	++	+	+
3	normal pressure glaucoma	42, M	4 weeks	+	++	+	++	++	++	++
4	idiophatic optic neuritis	41, M	4 weeks	++	++	+	+	++	+	++
5	idiophatic optic neuritis	38, F	4 weeks	++	++	+	+/-	+	+/-	+
6	ischemic optic neuritis	52, F	4 weeks	++	++	++	+	+/-	+	++
7	ischemic optic neuritis	58, F	4 weeks	++	++	+	++	++	+	++

Values are expressed as improvement with reference to the values before the treatment with NGF: "-" = constant or worsening; "+/-" = improvement < 19 %; "+" = improvement between 11 % and 25 %; "++" = improvement between 26 % and 50 %; "+++" = improvement between 51 % and 75 %; "++++" = improvement higher than 75 %;

1) ERG = electroretinogram;

2) OBF = blood flow of central retinal artery;

3) OCT = thickness of the nervous fiber layer.

Studies on the effect of NGF for vitreous body pathologies

[0058] A balanced saline solution containing 250 μg/ml of NGF was administrated three times a day for 4 weeks to 4 patients affected by myiodesopsia due to the presence of mobile vitreous bodies. After 4 weeks of treatment all the patients recognised a symptomatology amelioration.

Studies on the effect of NGF for choroideal pathologies

[0059] To evaluate the effect of external ophthalmic administration of NGF on choroideal pathologies an animal model of auotoimmune uveitis, obtained by administration of S retinal antigen to rats, was used. A group of animals was treated every two hours with one drop of NGF in the form of collyrium at a concentration of 200 μg/ml in saline balanced solution. After 4 weeks of treatment the lesions over vitreous body-retina in animals treated with NGF in the form of collyrium were compared to those present in animals treated with saline solution. In all the animals treated with NGF a reduction of tissues lesions was clearly visible.

[0060] The present invention was described with reference to specific embodiments thereof but it to be is intended that variations and modifications can be made by those skilled in the art without departing from the scope thereof as defined in the appended claims.

Claims

1. Use of nerve growth factor (NGF) for the production of an ophthalmic preparation for administration over the intact ocular surface for the therapy and/or the prophylaxis of pathologies affecting the sclera, ciliary bodies, crystalline lens, retina, optic nerve, vitreous body and/or the choroidea, wherein said ophthalmic preparation contains from 200 to 500 μg/ml of NGF.

2. Use according to claim 1, wherein said ophthalmic preparation is in the form of a solution or a suspension, an ointment, a gel or a cream in a pharmaceutically acceptable ophthalmic carrier or in the form of an ocular erodible insert or a polymeric membrane "reservoir" system to be placed in the conjunctival sac or it is added to a local bandage together with a therapeutic contact lens.

3. Use according to claims 1 or 2, wherein said pathologies have trophic, post-traumatic, infective, post-surgical, autoimmune, dystrophic, degenerative or post-inflammatory origin, or are originated by laser treatment.

4. Use according to any one of claims 1-3, wherein said ophthalmic preparation is in the form of an ophthalmic solution.

5. Use according to claim 4, wherein said ophthalmic solution contains from 200-250 μg/ml of NGF.

6. Use according to any one of claims 1-5, wherein the NGF in said preparation is in association with one or more other active ingredients and/or it is conjugated with a carrier molecule.

7. Use according to any one of the preceding claims wherein said NGF is of murine or of human origin, or it is human recombinant NGF.

Patentansprüche

1. Verwendung von Nervenwachstumsfaktor (NGF) zur Herstellung einer ophthalmischen Präparation zur Verabreichung über die intakte Augenoberfläche für die Therapie und/oder die Prophylaxe von Pathologien, welche die Lederhaut, Ziliarkörper, kristalline Linse, Retina, den Sehnerv, Glaskörper und/oder die Aderhaut betreffen, wobei die ophthalmische Präparation 200 bis 500 μg/ml NGF enthält.

2. Verwendung nach Anspruch 1, wobei die ophthalmische Präparation in Form einer Lösung oder einer Suspension, einer Salbe, eines Gels oder einer Creme in einem pharmazeutisch annehmbaren ophthalmischen Träger oder in Form eines vom Auge abbaubaren Einsatzes oder eines Polymer-Membran-"Reservoir"-Systems zur Platzierung im Bindehautsack vorliegt oder einer lokalen Bandage zusammen mit einer therapeutischen Kontaktlinse zugegeben wird.

3. Verwendung nach Anspruch 1 oder 2, wobei die Pathologien trophischen, post-traumatischen, infektiösen, post-chirurgischen, autoimmunen, dystrophischen, degenerativen oder post-entzündlichen Ursprung haben oder von einer Laserbehandlung herrühren.

4. Verwendung nach irgendeinem der Ansprüche 1 bis 3, wobei die ophthalmische Präparation in Form einer ophthalmischen Lösung vorliegt.

5. Verwendung nach Anspruch 4, wobei die ophthalmische Lösung 200 bis 250 µg/ml NGF enthält.

6. Verwendung nach irgendeinem der Ansprüche 1 bis 5, wobei der NGF in der Präparation in Assoziation mit einem oder mehreren anderen aktiven Bestandteil(en) vorliegt und/oder mit einem Trägermolekül konjugiert ist.

7. Verwendung nach irgendeinem der vorhergehenden Ansprüche, wobei der NGF von der Maus oder dem Menschen stammt oder humaner rekombinanter NGF ist.

Revendications

1. Utilisation du facteur de croissance nerveuse (NGF) pour la production d'une préparation ophtalmique à administrer sur la surface oculaire intacte à titre de thérapie et/ou de prophylaxie de pathologies affectant la sclère, les corps ciliaires, le cristallin, la rétine, le nerf optique, le corps vitré et/ou la choroïde, dans laquelle ladite préparation ophtalmique contient de 200 à 500 µg/ml de NGF.

2. Utilisation selon la revendication 1, dans laquelle ladite préparation ophtalmique est sous la forme d'une solution ou d'une suspension, d'une pommade, d'un gel ou d'une crème dans un excipient ophtalmique pharmaceutiquement acceptable ou sous la forme d'un insert oculaire dégradable ou d'un système "réservoir" à base d'une membrane polymère à placer dans le sac conjonctival ou qui est ajouté à un bandage local conjointement avec une lentille de contact thérapeutique.

3. Utilisation selon la revendication 1 ou 2, dans laquelle lesdites pathologies ont une origine trophique, post-traumatique, infectieuse, post-chirurgicale, auto-immunitaire, dystrophique, dégénérative ou post-inflammatoire, ou sont provoquées par un traitement laser.

4. Utilisation selon l'une quelconque des revendications 1 à 3, dans laquelle ladite préparation ophtalmique est sous la forme d'une solution ophtalmique.

5. Utilisation selon la revendication 4, dans laquelle ladite solution ophtalmique contient de 200 à 250 µg/ml de NGF.

6. Utilisation selon l'une quelconque des revendications 1 à 5, dans laquelle le NGF dans ladite préparation est en association avec un ou plusieurs ingrédients actifs et/ou est conjugué avec une molécule porteuse.

7. Utilisation selon l'une quelconque des revendications précédentes dans laquelle ledit NGF est d'origine murine ou humaine, ou est un NGF recombinant humain.